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prdm1a and *olig4* act downstream of Notch signaling to regulate cell fate at the neural plate border

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ABSTRACT

The zinc finger domain transcription factor *prdm1a* plays an integral role in the development of the neural plate border cell fates, including neural crest cells and Rohon–Beard (RB) sensory neurons. However, the mechanisms underlying *prdm1a* function in cell fate specification is unknown. Here, we test more directly how *prdm1a* functions in this cell fate decision. Rather than affecting cell death or proliferation at the neural plate border, *prdm1a* acts explicitly on cell fate specification by counteracting *olig4* expression in the neighboring interneuron domain. *olig4* expression is expanded in *prdm1a* mutants and *olig4* knockdown can rescue the reduced or abrogated neural crest and RB neuron phenotype in *prdm1a* mutants, suggesting a permissive role for *prdm1a* in neural plate border-derived cell fates. In addition, *prdm1a* expression is upregulated in the absence of Notch function, and inhibiting Notch signaling fails to rescue *prdm1a* mutants. This suggests that *prdm1a* functions downstream of Notch in the regulation of cell fate at the neural plate border and that Notch regulates the total number of progenitor cells at the neural plate border.

Introduction

The development of the nervous system involves a complex series of inductive interactions followed by the combinatory action of transcription factors to specify explicit cell fates. The embryonic neural plate is induced from the ectoderm in the presence of low levels of BMP signaling with opposing action from Shh required for ventral neural tube patterning. The neural plate border (NPB), which lies between the neural and non-neural ectoderm, requires an intermediate level of BMP signaling for its formation (Ahrens and Schlosser, 2005; Mancilla and Mayor, 1996; Rossi et al., 2008). Rohon–Beard sensory neurons, neural crest cells (NCCs), and placodal cell populations form at this junction in a medial to lateral orientation, respectively. Rohon–Beard (RB) sensory neurons are primary sensory neurons localized in the dorsal spinal cord that mediate proprioceptive mechanosensory information and are required for the touch response in zebrafish and *Xenopus* embryos (Lamborghini, 1980). Neural crest cells are a transient embryonic cell population that gives rise to neurons and glia of the peripheral nervous system, melanocytes, and cartilage of the face. Placodal populations form at the lateral-most edge of the NPB giving rise to neurons, and along with neural crest cells, contribute to the cranial ganglia (Ahrens and Schlosser, 2005; Schlosser, 2006). NPB cell fate specification requires the combinatory action of many transcription factors including *prdm1a*, *zic* genes, *pax3/7*, *msx1/2*, *and dlx* family members (reviewed in Meulemans and Bronner-Fraser, 2004). Further, specification of the different types of cells that form at the NPB requires action of a different set of transcription factors, including *foxd3*, *snail1*, *slug (snail2)*, *twist*, *ap-2*, and *sox9/10* for neural crest cells (Meulemans and Bronner-Fraser, 2004); *dlx* genes, *msx* genes, *neurog1*, *neuroD* and *islet1* for RB neurons (Rossi et al., 2009); and *dlx* genes, *six1*, *eya1* and *pax* genes for placodal cells (Schlosser et al., 2008). Expression analysis of these factors suggests that early in development, there is significant overlap between the NPB and placodal domain (marked by *dlx3b*), while there is little to no overlap between the NPB and the neural plate itself. Previous lineage tracing data suggests that neural crest cells and RB sensory neurons arise within the same domain at the NPB, while placodal cells arise from a domain lateral to the NPB (Cornell and Eisen, 2002; Schlosser, 2006).

In addition to BMP signaling, Delta/Notch signaling also regulates the specification of neural crest and RB neuron precursors. Notch receptors are expressed in a broad domain within the neural plate and non-neural ectoderm, while the *delta* ligand genes are expressed in primary neuron domains (Appel and Eisen, 1998; Hsiao et al., 2007). In the absence of Notch signaling, as occurs in the zebrafish *mind bomb (mib)* and *deltaA* mutants, there is an excess of all primary neurons, including RB sensory neurons and interneurons, and a concomitant reduction in the number

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of trunk neural crest cells (Cornell and Eisen, 2000; Itoh et al., 2003; Jiang et al., 1996). In *Xenopus*, overexpression of the Notch intracellular domain (NICD), which constitutively activates Notch signaling, at the end of gastrulation increases the expression of the neural crest markers Xslug and *foxd3*, suggesting that Notch signaling is required for NCC induction (Glavic et al., 2004). Blocking Notch with DAPT, a gamma-secretase inhibitor, produces a neurogenic phenotype with an increase in the number of primary neurons (Geling et al., 2002). These findings suggest that lateral inhibition between neurons and NCCs is perturbed such that neuronal fate is promoted at the expense of neural crest fate, at least in the trunk region (Cornell and Eisen, 2005). Notch signaling is also required for neurogenesis within the neural plate itself. As the neural plate domain eventually folds into the neural tube, Notch is required in the neural epithelium for V2 and dIL interneuron populations (Batista et al., 2008; Mizuguchi et al., 2006).

Prdm1a is a PR/SET domain, zinc finger domain transcription factor that functions as a master cell fate regulator in many cell types. *Prdm1a* (*Blimp-1* in mouse) has been implicated in differentiation of plasma cells from B-cells (Shaffer et al., 2002; Shapiro-Shelef et al., 2003) by regulating cell proliferation (Lin et al., 1997; Lin et al., 2002). A conditional knockout of *Blimp-1* in mouse using *Sox2*-cre demonstrates that *prdm1a* is required for the development of the posterior forelimb, caudal pharyngeal arches, secondary heart field, and sensory vibrissae (Robertson et al, 2007). In zebrafish, *prdm1a* functions at multiple stages during development, including gastrulation, formation of head structures and fin development (Mercader et al., 2006; Wilm and Solnica-Krezel, 2005), and in a Hedgehog-regulated switch between slow twitch and fast twitch muscle development (Baxendale et al., 2004 (Liew et al., 2008; von Hofsten et al., 2008). In addition to these roles, *prdm1a* is required for neural crest and RB neuron cell fate. *prdm1a* mutant embryos have a decreased number of trunk neural crest cells, a complete loss of RB sensory neurons (Artinger et al., 1999; Roy and Ng, 2004; Hernandez-Lagunas et al., 2005) and a loss of neural crest-derived ceratobranchial cartilage within the craniofacial skeleton (Birkholz et al., 2009). *prdm1a* is expressed at midgastrulation in the NPB and continues to be expressed until the 6-somite stage when expression is downregulated, but remains in the posterior pharyngeal arches. (Hernandez-Lagunas et al., 2005; Wilm and Solnica-Krezel, 2005; Birkholz et al., 2009).

olig4 (*Olig3* in mouse) is a member of the basic helix–loop–helix family of transcription factors expressed within the interneuron domain of primary neurons, and has been shown to regulate interneuron fate in both mouse and zebrafish. *Olig3* is expressed in the p1–p3 dorsal interneuron domain of the mammalian spinal cord and mutations in *Olig3* eliminate p2–3 interneurons and severely reduce the d1 population (Muller et al., 2005). Interestingly, in contrast to *olig4* mutants in zebrafish, neural crest cells are unaffected in these mouse mutants. Knock down of *olig4* in zebrafish results in a loss of interneurons and expansion of the neighboring neural crest and RB neuron domain (Filippi et al., 2005; Tiso et al., 2009), while overexpression reduces neural crest cell number. These data suggest that *olig4* normally acts as a negative regulator of NPB cell fates. Interestingly, *olig4* has been shown to be downstream of both BMP and Wnt signaling in the specification of dorsal interneurons (Filippi et al., 2005; Zechner et al., 2007). In addition, in zebrafish *olig4* is downstream of Notch, as knockdown of *olig4* in Notch-deficient embryos rescues the loss of neural crest cells. This suggests that *olig4* expression must be reduced for Notch signaling to specify cell fate (Filippi et al., 2005).

Here, we have further dissected the function of *prdm1a* and *olig4* in NPB cell fate decisions. We show that *prdm1a* acts to regulate cell fate at the NPB by counteracting *olig4* expression, but not by regulating cell death or proliferation. In addition, *prdm1a* expression is upregulated in the absence of Notch function, and we are not able to rescue *prdm1a* mutants by blocking Notch with DAPT. This suggests that *prdm1a* functions downstream of Notch in the regulation of cell fate at the neural plate border.

Materials and methods

Animals

The zebrafish were maintained according to Westerfield (1993) and staged by hours post fertilization (hpf) and morphology according to Kimmel et al. (1995). The zebrafish *prdm1a*^{m805}, *deltaA*, and *mind bomb* mutants have been described previously (Artinger et al., 1999; Hernandez-Lagunas et al., 2005; Rossi et al., 2009; Birkholz et al., 2009; Itoh et al., 2003). Single embryo phenotyping and genotyping in *prdm1a* clutches was performed for cell death, proliferation and rescue experiments as previously described (Olesnicky et al., 2010; Rossi et al., 2009). For *deltaA* genotyping, we used primers and protocols provided by ZIRC, *mib*^{m132} genotyping is as described in (Itoh et al., 2003).

Embryo manipulation and analysis

Whole-mount in situ hybridization was adapted from Thisse and Thisse (1998). Fluorescent ISH was performed using the protocol described in (Pineda et al., 2006), in which a DIG-conjugated probe was developed using a fast red kit (Sigma F4648) and a fluorescein-conjugated probe was developed using a TSA kit (Perkin Elmer NEL741). Immunohistochemistry was performed as described (Ungos et al., 2003) and the following antibodies were used: HNK-1 antibody (Sigma) at a 1:1000 dilution; islet1/2 (39.4D5) at 1:200 (Developmental Studies Hybridoma Bank); anti-phosphohistone-H3 (Upstate) at 1:500; and Alexa568 goat anti-mouse at 1:750. Confocal microscopy was performed on Leica TCS SP5 II laser scanning confocal using LAS AF software. Apoptosis was determined by TUNEL labeling using fluorescein-dUTP (TMR-Red, Roche). Total cells expressing pH3 and TUNEL were counted and compared to wildtype. 6–10 ng of *prdm1a* Morpholino (5'-TGGTGTCATACCTCTTTGGAGTCTG-3') was injected into the 1 cell stage for knockdown and rescue as previously described (Hernandez-Lagunas et al., 2005). 6–10 ng of *olig4* Morpholino (MO) was injected into 1cell stage embryos as described in (Filippi et al., 2005). The standard control MO 5′-CCT CTT ACC TCA GTT ACA ATT TAT A 3′ was injected at 10 ng. At least three experiments in separate clutches were done for each experimental condition.

Analysis of *prdm1a* mutant rescue with *olig4* MO injections was done as follows: Neural crest cell rescue was defined as the presence of NCC in 7 or more somites, since *prdm1a* mutants rarely have NCC in that number of somites. RB sensory neurons were counted across 20 segments at both 24 hpf in the tg[*neurog1*::gfp] line or at 48 hpf using HNK-1 immunohistochemistry. Pigment cells were scored as rescued if there was pigment on the yolk (which *prdm1a* mutants rarely have) and an increase in pigment cells on the dorsal aspect of the embryo.

DAPT treatments were performed on wildtype or *prdm1a*−/− or morphant embryos. 100 μM, 200 μM, or 1% DMSO in embryo media was applied to embryos at 60% epiboly stage with holes poked in the chorions. Embryos were fixed at the tailbud to 2 somite stage in 4% PFA at 4 °C overnight. Chorions were then fully removed and embryos were dehydrated in MeOH.

Results

prdm1a expression overlaps with pax3 but not olig4 at the neural plate border

To investigate the relationship between *prdm1a* and other cell fate regulators at the NPB, we performed double fluorescent *in situ* hybridization with markers of the NPB and neural tissue between 90% epiboly and the 2 somite stage. Previous work has shown that *prdm1a* overlaps extensively with the non-neural ectoderm marker *dlx3b* at gastrulation stages and 90% epiboly, but not with the neural markers *sox19a* or *sox3*. Expression of *prdm1a* and *dlx3b* further resolves into individual domains beginning at tailbud stage (Rossi et al., 2009). *pax3*

and *pax7* act to specify the NPB and are required for NC development in other vertebrates (Basch et al., 2006). We examined the expression of the NPB marker *pax3* and the interneuron marker *olig4* in comparison to *prdm1a*. At 90% epiboly through tailbud, we observe overlap of expression of *prdm1a* with the NPB marker *pax3* in the anterior region of the embryo. By the 2-somite stage, expression of *prdm1a* and *pax3* resolves into separate domains (Figs. 1A–C). *pax3* expression is not altered in *prdm1a* mutant embryos (see Fig. 3), indicating that *prdm1a* does not regulate *pax3* expression. This observation is important as it allows us to utilize *pax3* in other experiments to identify the NPB domain in *prdm1a* mutant embryos.

olig4 (previously named *olig3*) is expressed within the intermediate domain of primary neurons, and has been shown to regulate interneuron fate. Embryos in which Olig4 has been knocked down exhibit a loss of interneurons and expansion of the neighboring NC and RB neuron domain (Filippi et al., 2005; Tiso et al., 2009). Since *prdm1a* is required in the neighboring neural crest and RB neuron domain, we sought to understand the relationship between these two transcription factors. To determine whether *prdm1a* expression overlaps with the interneuron domain, we examined the expression of *olig4* and *prdm1a* using double fluorescent *in situ* hybridization. Previous work has shown no overlap in the expression of *prdm1a* with pan neural plate markers (Rossi et al., 2009). Consistently, *prdm1a* did not overlap with *olig4* at any stage examined (Figs. 1D–F). Therefore, we conclude that *prdm1a* is expressed within the NPB but not within the neural plate/interneuron domain.

The olig4 domain is expanded in prdm1a mutant embryos

olig4 morphants have a phenotype opposite to that of *prdm1a* mutant embryos, exhibiting greater numbers of NCC and RB sensory neurons at the expense of interneurons, suggesting that both genes are required for establishing distinct cell fates within the NPB (Filippi et al., 2005). To assay the fate of cells after *prdm1a* loss, we examined the expression of both the non-neural ectoderm marker *dlx3* and interneuron domain marker *olig4* in *prdm1a* mutant embryos and *prdm1a* morphant embryos at 95% epiboly and tailbud stage. In wildtype embryos at both stages, a 2–4 cell gap exists between the *olig4* and *dlx3b* expression domains, corresponding to the *prdm1a* expression domain (Figs. 2A, D). Upon loss of *prdm1a* in the morphant or mutant embryos, this gap between *olig4* and *dlx3b* expression domains is lost (Figs. 2B, C, E, F, arrows as compared to wildtype). These data suggest that *prdm1a* normally acts to repress either *dlx3b* or *olig4* to maintain a zone of cells fated to become NC and RB sensory neurons. Previous studies have shown that *dlx3b* expression is reduced and not expanded in the posterior domain (Artinger et al., 1999; Rossi et al., 2009). This suggests that *prdm1a* might normally repress *olig4* expression and that *olig4* expression may expand into the *prdm1a* domain in *prdm1a* mutant embryos. To quantify this increase, we examined *olig4* expression at 2-somite stage in whole mount embryos and counted the number of *olig4* expressing cells along the anterior-posterior axis (Fig. 2I). We observed a 2–3 cell increase in the anterior region and a slight increase in the posterior domain of *olig4* expression in the *prdm1a* morphant embryos compared to wild type controls. Using confocal imaging to optically section in the Z-axis, we additionally show that this change in the *olig4* expression domain is a change in the medial lateral domain of the neural plate and not an expansion into deeper cell layers (Figs. 2J, K). Together, these data indicate that *olig4* expands into the *prdm1a* domain in the absence of *prdm1a* function (Fig. 2H; 2 somite uninjected, n = 6, *prdm1a* MO, n = 8). However, at earlier tailbud stages, we did not observe an increase

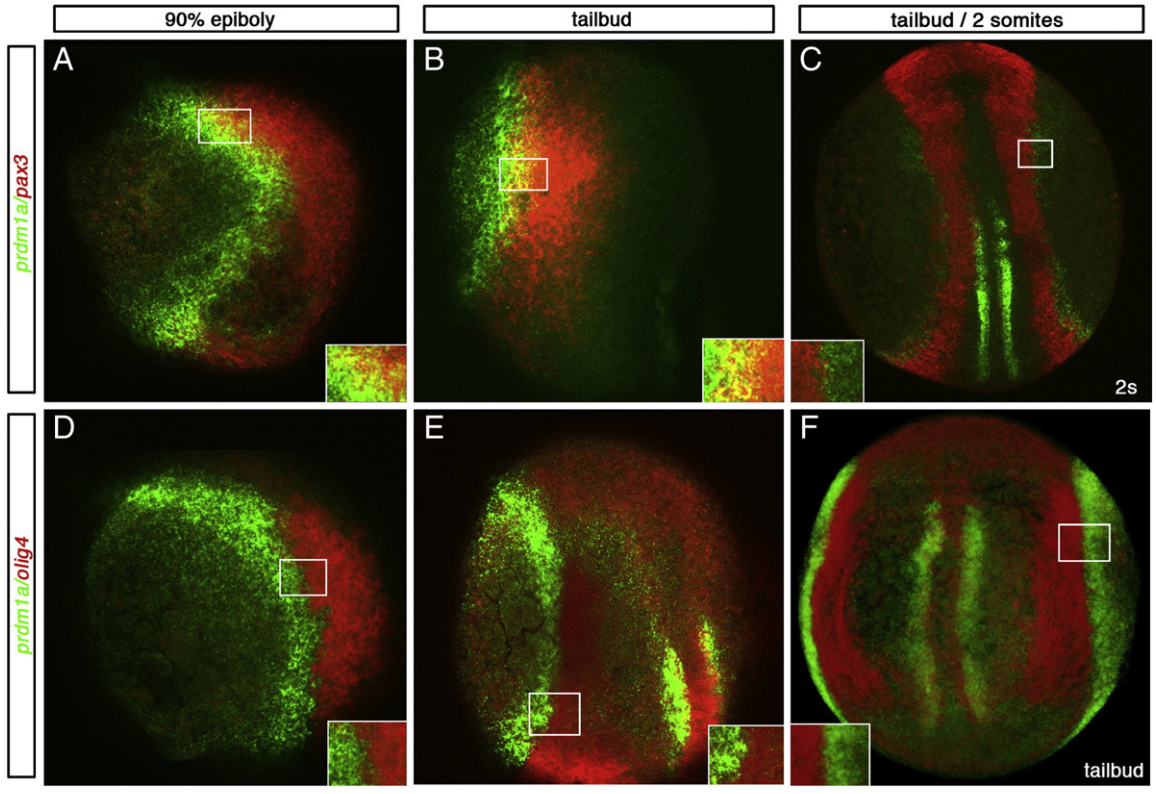

Fig. 1. Double fluorescent *in situ* hybridization of *prdm1a* with *pax3* and *olig4*. Dorsal and lateral views of 90% epiboly, tailbud and 2 somite stage (9 hpf–11 hpf) confocal micrographs in a single z-stack unless otherwise noted. Insets show higher resolution confocal images for each boxed area. (A–C) *prdm1a* in green and *pax3* in red and (D–F) *prdm1a* in green and *olig4* in red. A lateral view, dorsal to the right, of an embryo at the end of gastrulation (90% epiboly) and lateral view at tailbud exhibit overlap between *prdm1a* and *pax3* (A, B; yellow). By 11hpf, dorsal view of a 2 somite stage embryo, projected image shows the domains are distinct (C). Even at the earliest stages examined, *olig4* is distinct from *prdm1a*. Lateral view of 90% epiboly and dorsal lateral view of a tailbud stage embryo show no overlap in expression (D, E). At tailbud stages shown in a dorsal projected view (F), no overlap is seen in the dorsal domain or the medial adaxial domain.

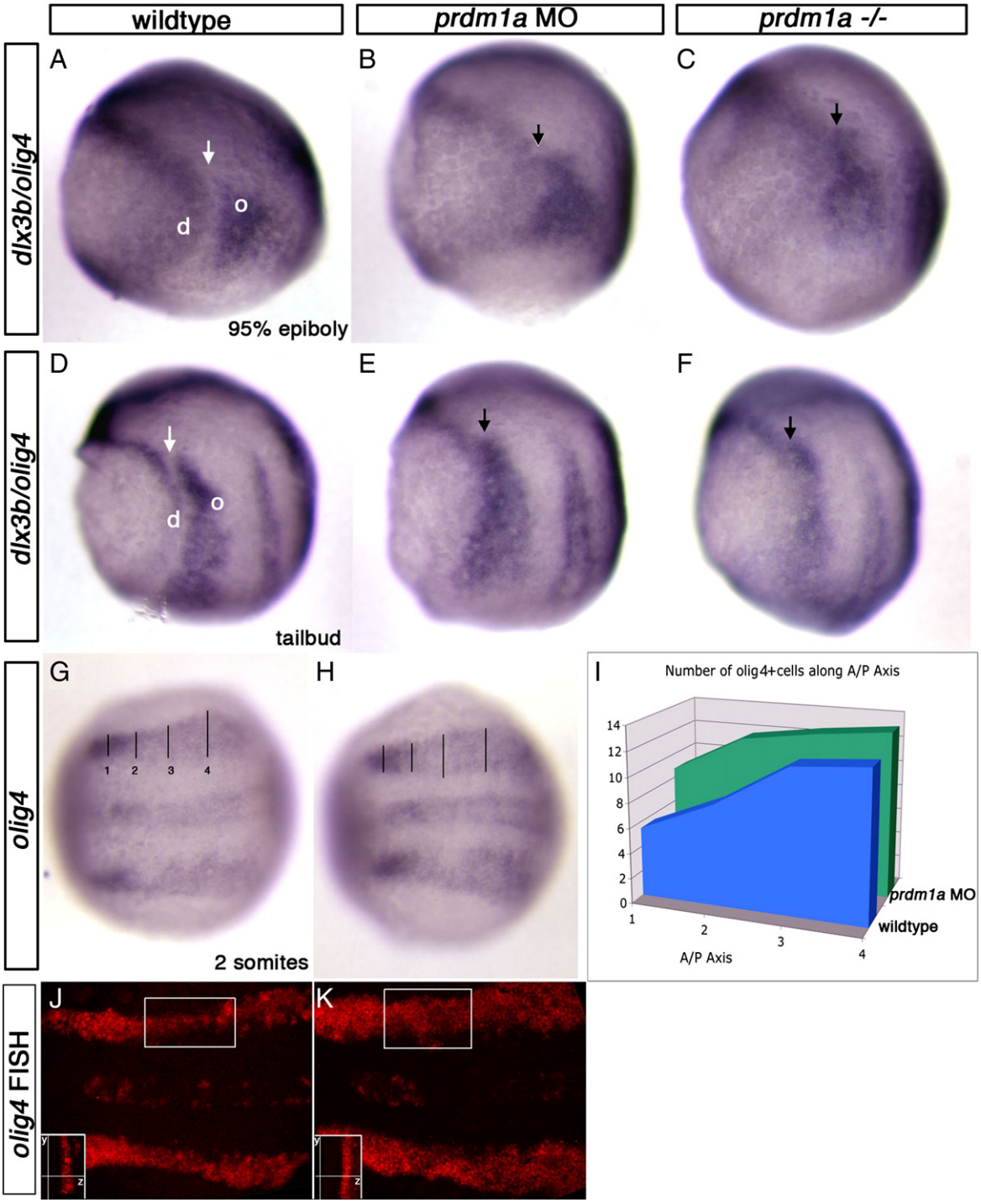

Fig. 2. Loss of *prdm1a* results in expansion of the *olig4* domain. Dorsal and lateral views of wildtype, *prdm1a* morphant and *prdm1a* mutant embryos at 95% epiboly, tailbud and 2 somite stage. (A–C) Lateral view, dorsal to the right. There is a gap between the expression domains of *dlx3b* (d) and *olig4* (o) in wildtype embryos that corresponds to the domain of *prdm1a* expression (arrow). When there is no *prdm1a* expression, the gap is lost (black arrow). Expression of *dlx3b* (left of arrow) in the non-neural ectoderm domain and *olig4* (right of arrow) in the interneuron domain shifts to where there is no gap in *prdm1a* deficient embryos (arrows). The gap represents the position of the *prdm1a* domain. (D–F) Similar results are observed at the tailbud stage. (G–I) *olig4* expression at the 2 somite stage in embryos that are mounted dorsally to count the number of cells in the *olig4* domain. *olig4* expression in the lateral interneuron domain and medial motor neuron domain (H) is expanded in *prdm1a* morphant embryos, as compared to wildtype embryos (G). (I) Cells were counted in four anterior/posterior (A/P) positions across the medial–lateral extent of the *olig4* domain in wildtype (blue) and *prdm1a* morphant embryos (green) showing an expansion of the number of cells expressing *olig4* especially in the anterior region (positions 1 and 2). (J,K) Fluorescent *in situ* hybridization (FISH) showing *olig4* expression in *prdm1a* morphants is expanded in the medial lateral domain. However, expression does not expanded into the deeper cell layers, shown in a Z-plane confocal section (insets). Insets, boxed area observed at higher magnification in the Z-plane showing the same number of pixels in both uninjected and *prdm1a* morphants. y, y plane, anterior–posterior axis; z, z plane, dorsal–ventral axis.

in the size of the *olig4* domain in *prdm1a* mutants, suggesting that while the initiation of *olig4* expression is normal in *prdm1a* mutant embryos, the subsequent spatial refinement of *olig4* expression requires Prdm1a function.

On the non-neural ectoderm side of the neural plate border, *dlx3b* expression is required for the development of RB sensory neurons and neurogenic placodes. Neurogenic placodes form as thickenings in the non-neural ectoderm next to the domain of NCCs, and form derivatives

of the cranial and sensory organs (Nechiporuk et al., 2007). Previous work has shown that, in the absence of Prdm1a function, *dlx3b* is absent in the caudal domain, but does not expand into the NPB domain (Artinger et al., 1999). Since the neighboring interneuron domain marked by *olig4* is affected in *prdm1a* mutants, we wanted to determine if the placodal domain or derivatives are also affected in *prdm1a* mutants. Placodal development was not changed in *prdm1a* mutant embryos as assessed by *eya1* expression at the 2 somite stage (Supplemental Figure S1). There was also no change in the placodally-derived otic vesicle at the 10-somite stage, as demarked by *pax2a* expression (Supplemental Figure S1). Therefore, we conclude that the placodal domain is unaffected following *prdm1a* loss.

prdm1a acts to regulate cell fate, not cell proliferation or death, at the neural plate border

The results above indicate that *prdm1a* represses expression of the neighboring *olig4* domain to maintain NPB cell fate. However, because *prdm1a* mutant embryos show decreased numbers of NPB derivatives, it is also possible that *prdm1a* regulates proliferation or death of NPB cells, similar to its role in the mouse immune system (Lin et al., 1997) and the zebrafish posterior pharyngeal arch region (Birkholz et al., 2009). To test the possibility that *prdm1a* mutants lack neural crest and RB neurons due to apoptosis within these cell types, we used TUNEL to assess rates of cell death at the NPB or dorsal neural tube of *prdm1a* mutants between 80% epiboly and the 6 somite stage within the *pax3* expression domain. There is no change in the level of apoptosis at the NPB or in the dorsal neural tube of *prdm1a* mutant embryos compared to controls at either stage of development (data not shown). We found an average of 7.4 apototic cells at the 2 somite stage in both wildtype/heterozygotes (n = 29) and *prdm1a*$^{-/-}$ (n = 12); and an average of 5.3 apototic cells at the 6 somite stage in wildtype/heterozyogotes (n = 43) and 5.6 in *prdm1a*$^{-/-}$ (n = 15). These results show that the loss of NPB derivatives in *prdm1a* mutants is not due to an increase in cell death.

We next examined the levels of cell proliferation between 95% epiboly and the 6 somite stage to determine if *prdm1a* is required for proliferation of NPB progenitors. We used a phosphohistone H3 (pH3) antibody to identify proliferating cells and counted pH3^{+} cells within the *pax3* expression domain observed by *in situ* hybridization, which overlaps with the *prdm1a* domain at the NPB until the 2 somite stage (Fig. 1). At 95% epiboly through the 2 somite stage, there was no significant difference in the number of proliferating cells in control embryos compared to *prdm1a* mutant embryos (Fig. 3). At 95% epiboly, uninjected embryos had an average of 39.9 proliferating cells (n = 9) and *prdm1a* MO had an average of 43.3 (n = 8) within the NPB. At the 2 somite stage, uninjected embryos had an average of 5.9 (n = 10) and *prdm1a* MO had an average of 6.3 proliferating cells (n = 12). However, at the 6 somite stage, there is a significant increase in the number of mitotic cells within the region of the cranial neural crest in *prdm1a* MO embryos; uninjected embryos averaged 5.9 pH3^{+} cells at the 6 somite stage (n = 8), while *prdm1a* MO embryos had an average of 10.0 (n = 10; Student's t-test, p = 0.003). This is consistent with the observation that while *prdm1a* mutants have reduced cranial neural crest cells early in development, the number of cranial NCCs recovers to near normal levels by the 5–10 somite stage, as assessed by *snail2* and *dlx2* expression (Artinger et al, 1999). This suggests that the remaining neural crest cells in the cranial region of *prdm1a* mutant embryos proliferate to compensate for the earlier reduction in cranial NCCs. To further assess the role of proliferation in the *prdm1a* phenotype, we inhibited proliferation with Aphidicolin and HydroxyUrea from 70% epiboly to 1 somite, but this did not phenocopy the *prdm1a* mutation with respect to loss of neural crest markers and RB-specific markers (not shown). We therefore conclude that *prdm1a* is important specifically in regulation of cell fate decisions at the NPB and does not function by controlling cell death or proliferation of NPB progenitors.

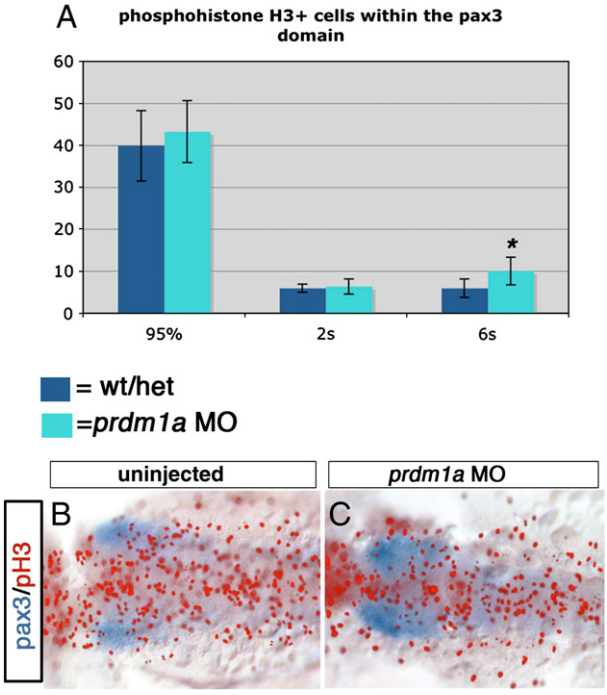

Fig. 3. Quantification of cell proliferation in *prdm1a* morphants. (A) Quantification of the pH3-expressing cells in wildtype and *prdm1a* morphant embryos at 95%, 2 somite stage and 6 somite stage: morphants in green, wildtype in blue. There is a significant increase in cell proliferation at the 6 somite stage, average of 5.9 pH3^{+} cells in wildtype (n = 8) vs 10.0 in *prdm1a* mutants, n = 10; p < 0.003. (B, C) Dorsal view of *pax3* expression in blue and pH3 pseudo-colored in red at the 6 somite stage in wildtype and *prdm1a* morphant embryos.

olig4 knockdown can rescue the neural crest and Rohon–Beard sensory neuron phenotype of prdm1a mutants

If *prdm1a* and *olig4* interact to define the NPB region, we should be able to determine the interaction of these transcription factors by epistasis experiments. If *prdm1a* and *olig4* repress each other in neighboring domains, thereby promoting NPB and interneuron fates respectively, we would expect that removing *olig4* would promote NC and RB cell fate even in the absence of *prdm1a*. First, we confirmed the published Morpholino knockdown phenotype of *olig4* alone on neural crest cell fates. We found that *olig4* increased *foxd3* and *crestin* expression and pigment cell number (Supplemental Fig. 2). To determine if *olig4* knockdown can restore the depleted NC in *prdm1a* mutants, we injected *olig4* Morpholino into *prdm1a* mutant and wildtype embryos. Consistent with previous reports, *olig4* knockdown promoted NC and RB fate in wildtype embryos (data not shown). In addition, control Morpholino injected at the same concentration shows no phenotype or restoration of neural crest cells (data not shown). Knockdown of *olig4* in *prdm1a* mutant embryos rescued NCC specification as demonstrated by an increase in expression of *crestin* (26 of 29 embryos—89%—express crestin) and *sox10* (29 of 32 embryos—90%—express sox10) in *prdm1a* mutants injected with *olig4* MO (Figs. 4A–C). Overall pigment cells also increased compared to uninjected control embryos 93%—41/44 of *prdm1a* mutants injected with olig4 MO—have increased pigment; Figs. 4D–I). RB sensory neurons are also partially rescued, albeit to a lesser extent, as shown by the partial induction of *islet1/2* and HNK-1 (4 of 10 *prdm1a* mutants injected with *olig4* MO express HNK-1 and islet1 compared to *prdm1a* mutants without *olig4* MO; Figs. 4M–O). Uninjected or control MO-injected *prdm1a* mutants exhibit no expression of either marker (Fig. 4). These results show that *olig4*

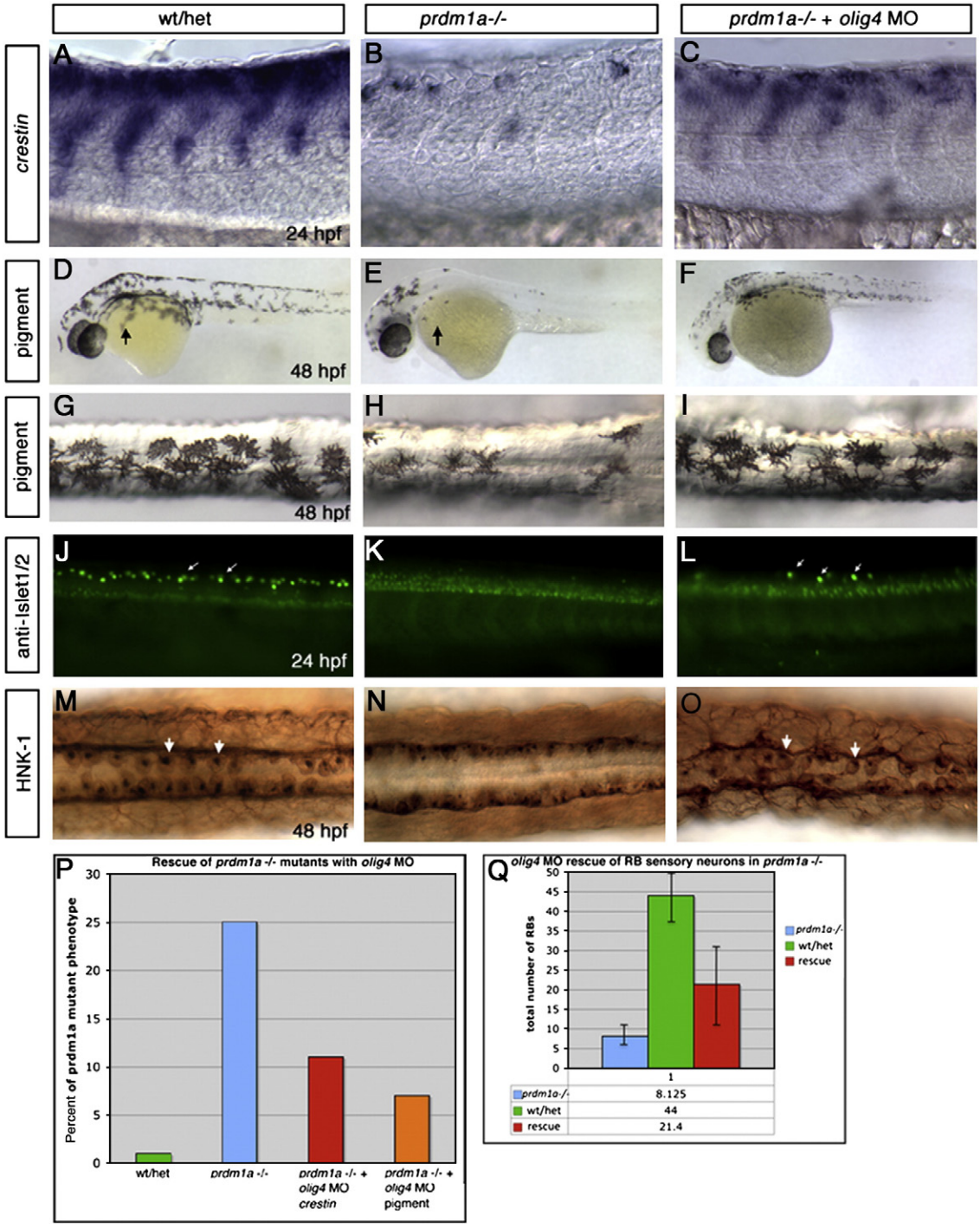

Fig. 4. Injections of *olig4* Morpholino rescues the *prdm1a* phenotype. (A–F, J–L) Lateral views and (G–I, M–O) dorsal view, anterior to the left of 24 and 48 hpf embryos. (A) *crestin* expression in wildtype or heterozygous embryo show a wildtype pattern of neural crest migration. (B) *prdm1a* mutant embryos have few neural crest cells, while injection of *olig4* MO rescues the neural crest cell deficit (C). (D, G) Low and high magnification of wildtype pigment pattern at 48 hpf compared to (E, H) *prdm1a* mutant embryos that have a reduction of pigment especially in the head and on the yolk (arrow). (F, I) *olig4* MO injection shows an increase in pigment cells overall and some migrate over the yolk. (J, M) Rohon–Beard sensory neuron expression with Islet1/2 or HNK-1 antibody at 24 hpf and 48 hpf respectively in wildtype embryo. (K, N) *prdm1a* mutant embryos have no RB sensory neurons but still have ventral expressing interneurons and motor neurons. (L, O) Injection of *olig4* MO into *prdm1a* mutant embryos increases the number of RB sensory neurons.

knockdown promotes the formation of NPB derivatives in the absence of *prdm1a* function. Thus, *prdm1a* is dispensable for NC and RB neuron specification, but is necessary to define the precise spatial domain of the NPB. Consequently, *prdm1a* plays a permissive rather than an instructive role in NPB specification and, thereby, in the cell fate specification of NPB derivatives. Moreover, the mutual repression of *prdm1a* and *olig4* is required for the establishment and refinement of distinct interneuron and NPB domains.

prdm1a is downstream of Notch signaling in neural crest and RB cell fate specification

Notch signaling plays an important role during generation of cell fate in the nervous system and, more specifically, in the fate determination step that defines neural crest and RB sensory neurons. In zebrafish Notch mutants and *Xenopus* embryos in which Notch signaling is over-expressed or knocked down, there is an increase in the number of

primary neurons at the expense of neural crest. Because *prdm1a* controls the fate of both of these populations, we wanted to determine how *prdm1a* expression is affected by Notch mutations and explore the epistasis of *prdm1a* and Notch signaling. First, we examined *prdm1a* expression in *mib* and *deltaA* mutants. In *mib* mutant embryos, which have complete blockage of Notch signaling, *prdm1a* expression was increased throughout the neural plate border domain and significantly expanded in the anterior domain (Fig. 5). In *deltaA* mutants, *prdm1a* expression was also increased within this domain but to a lesser degree (data not shown). As an alternate approach to genetically blocking Notch signaling, we also treated wildtype embryos with 100–200 μm DAPT at 60% epiboly until fixation at the 2 somite stage to inhibit Notch signaling specifically during the developmental window when neural crest and RB sensory neurons are being specified. We observed an increase in *prdm1a* expression in the NPB, but it was not statistically significant due to variability of treatment penetrance (Fig. 5). It is also possible that inhibition of Notch signaling must occur at earlier developmental stages to affect *prdm1a* expression completely. These results suggest that Notch signaling negatively regulates *prdm1a* expression and by doing so increases the progenitors available to produce more neurons. To test this, we performed epistasis experiments with Notch and *prdm1a*. We used DAPT to treat wildtype and *prdm1a* mutants as described. Embryos were examined for border domain markers (*olig4*/*dlx3b*), neural crest (by *foxd3* expression), and RB sensory neurons (by *huC* expression). We confirmed that treatment with DAPT in wildtype embryos increased the total number of primary neurons, as previously reported (Geling et al., 2002). However, even after application of DAPT to *prdm1a*−/− embryos, we did not observe a rescue of the border domain with restoration of the gap between *olig4* and *dlx3b* expression (Figs. 6A–D). In addition, RB sensory neurons were not recovered in *prdm1a* mutants (n = 39) as none exhibited *huC* expression at the 2 somite stage (Figs. 6E–H). All DAPT-treated *prdm1a* mutants (n = 27) had *foxd3* expression at similar levels to wildtype at 2 somite stage (Fig. 6 I–L). However, there was no difference between the *foxd3* expressing neural crest cells between DAPT- and vehicle-treated *prdm1a* mutant embryos, indicating that inhibition of Notch signaling was unable to restore the neural crest phenotype observed in *prdm1a* mutants. These results are consistent with Notch acting upstream of *prdm1a*.

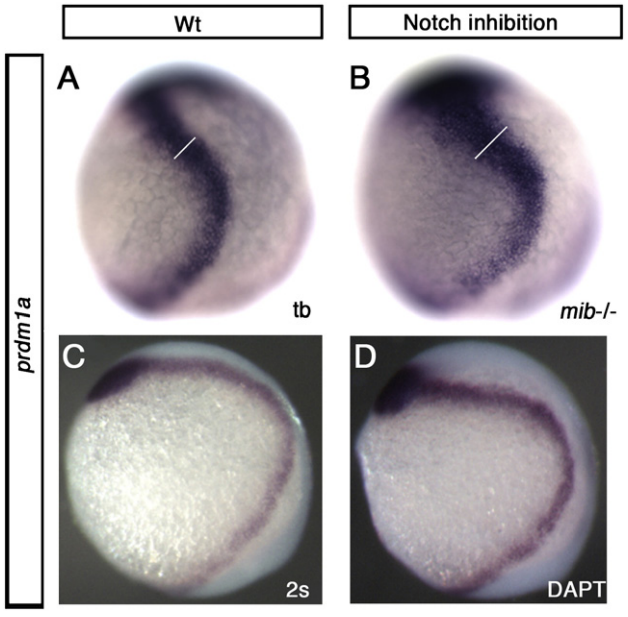

Fig. 5. *prdm1a* expression following Notch inhibition. Lateral views, anterior to the top, dorsal to the right of tailbud and 2 somite stage embryos. (A) *prdm1a* expression at tailbud in a wildtype embryo (B) and in the *mib*−/− background. The overall level of expression is increased and the border domain was significantly expanded in *mib* mutant embryos (white line indicates where anterior *prdm1a* expression was measured using Photoshop; WT average 1.56 arbitrary pixel units, n = 5; *mib* average 2.01 arbitrary pixel units, n = 6; Student's t-test, p = 0.03). (C) DMSO control treated wildtype and (D) embryo treated with DAPT a Notch inhibitor. *prdm1a* expression was increased overall when treated from 60% epiboly to 2 somite stage with DAPT.

Discussion

Here, we have further defined the function of *prdm1a* and *olig4* in NPB cell fate decisions. We show that *prdm1a* regulates cell fate at the NPB by counteracting *olig4* expression, not by regulating cell death or proliferation. In addition, *prdm1a* expression is upregulated in the absence of Notch function, and we are not able to rescue *prdm1a* mutants by blocking Notch signaling with DAPT. This suggests that *prdm1a* functions downstream of Notch in the regulation of cell fate at the neural plate border.

Prdm1a has been shown to play a role as a cell fate switch in a variety of developmental paradigms, including neural crest/RB neurons and interneuron cell fate. In *prdm1a* mutant embryos, we previously observed an increase in *islet1*-expressing cells within a ventral interneuron domain, supporting the idea that *prdm1a* promotes formation of neural crest and RB sensory neurons by repressing interneuron cell fate (Olesnicky et al., 2010). This is supported further by the observation that overexpression of *prdm1a* expands *sox10* and *islet1* expression specifically in the neural crest and RB neuron domain, respectively (Olesnicky et al., 2010). Studies in zebrafish pharyngeal arch development and in the immune system of mice show that, in these systems, *prdm1a* plays a role in regulating cell proliferation (Birkholz et al., 2009; Lin et al., 1997). However, the data we present here shows that *prdm1a* does not regulate cell proliferation during the NPB stage, but instead plays a role in regulating NPB cell fate. At later neural plate stages (the 6 somite stage), there is a slight upregulation of cell proliferation within the *pax3* domain. As the *pax3* domain does not correlate completely with the *prdm1a* domain, we cannot rule out the possibility that the upregulation in cell proliferation is within the neural plate domain and not at the NPB. Thus, *prdm1a* may act non-cell autonomously to influence cell proliferation or cell cycle progression of the neighboring interneuron domain of neural plate cells. It is clear that these domains, which are often thought of as autonomous at the developing neural plate and NPB are not distinct at early stages, but become resolved as development proceeds via mutual repression and other complex genetic interactions. Double fluorescent *in situ* hybridization has revealed overlapping domains of non-neural ectoderm, NPB and neural plate during gastrulation. This overlap implies that during the process of resolving the progenitor domains of interneurons, neural crest, and RB sensory neurons, NPB genes interact extensively with genes expressed in overlapping and neighboring domains. While it is clear that the domains refine over time to produce specific neurons with specific functions, we do not yet know whether cells move in between the domains and if so whether these cells acquire the fate specified by their new environment or move back to their initial domain. Additional experiments are required to address these questions. Interestingly, *prdm1a* seems to play a similar role in other regions of the developing nervous system. *Prdm1a* appears to function in the mouse retina by simultaneously promoting photoreceptor fate and repressing bipolar interneuron cell fate. In conditional knockout studies, *Prdm1a* (*Blimp1*) mutants exhibited fewer photoreceptors and more bipolar interneuron cells (Brzezinski et al., 2010; Katoh et al., 2010).

Previous studies in zebrafish suggest that *olig4* morphants have reduced interneuron cell number and increased numbers of neural crest cells and RB neurons (Filippi et al 2005). Because this is the opposite of the phenotype observed in *prdm1a* mutants, we were interested in understanding how these two transcription factors interact. Interestingly, *olig4* expression does not overlap with *prdm1a* expression even at early gastrulation stages, and its expression domain is expanded in *prdm1a* mutants. In addition, *olig4* Morpholino injection can restore NC

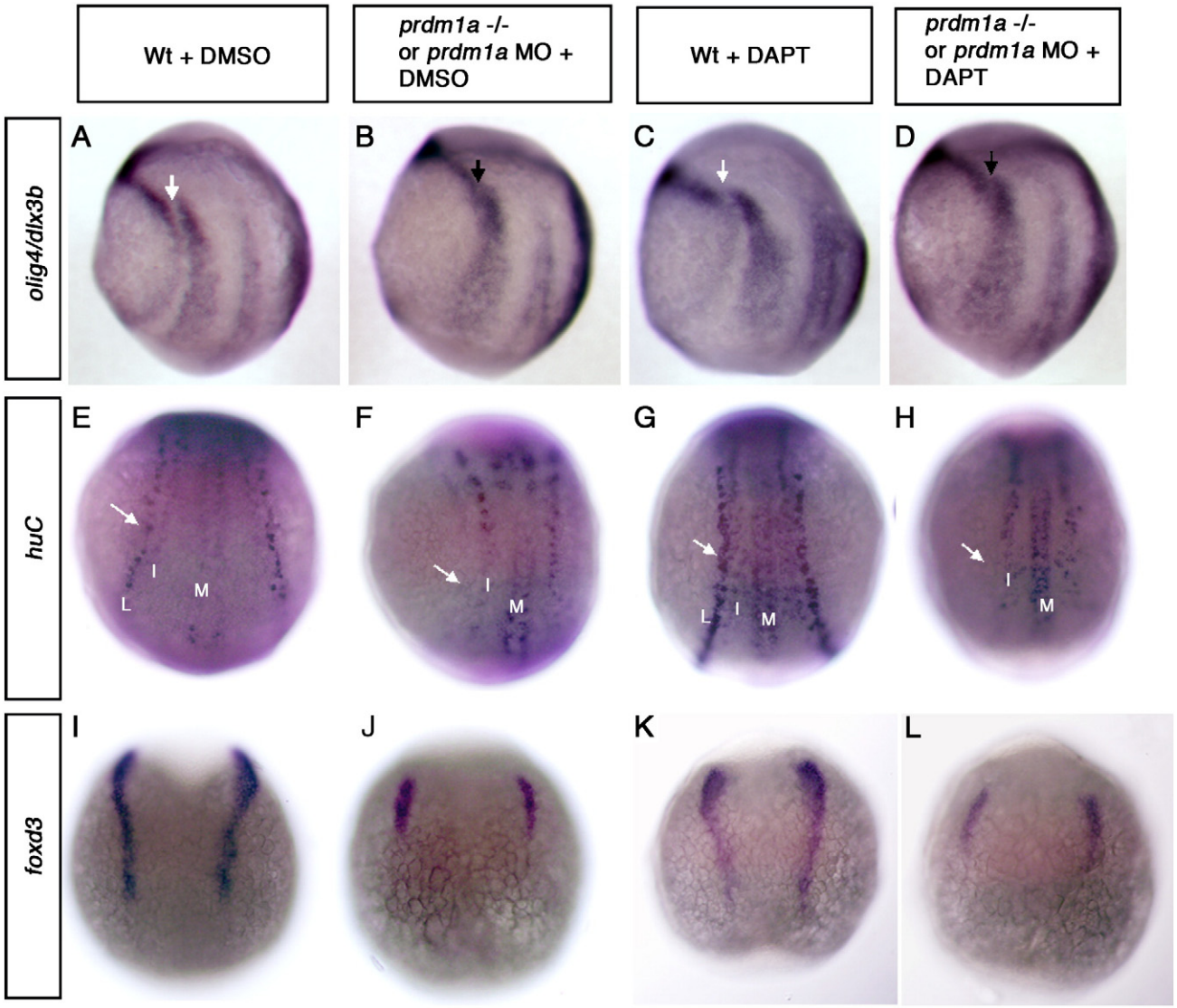

Fig. 6. Inhibition of Notch signaling cannot rescue the *prdm1a* mutant phenotype. (A–D) Lateral views and (E–L) dorsal view, anterior to the top of tailbud and 2 somite stage embryos. (A) Wildtype embryo showing expression of *olig4* and *dlx3b* and (B) *prdm1a* deficient embryos show no gap in expression of *olig4* and *dlx3b* at tailbud stage as observed in wildtype. With the addition of DAPT from 60% epiboly to tailbud, wildtype embryos (C) have a slightly wider gap corresponding to the *prdm1a* expression domain, while *prdm1a* mutant embryos maintain the gap in *olig4* and *dlx3b* expression after DAPT treatment (arrows). (E, G) Wildtype and *prdm1a* (F, H) mutant embryo at 2 somites; RB sensory neurons shown by the lateral expression of *huc* (arrows) were not recovered in *prdm1a*−/− embryos following DAPT treatment and look similar to DMSO control treated mutants. Wildtype and mutant embryos treated with DAPT show an increase in the overall number of primary neurons (L, I, M) as previously reported. (I–L) Similarly *foxd3* expressing neural crest cells are not increased in *prdm1a* mutant embryos following DAPT treatment. L, Lateral Rohon–Beard sensory neurons; I, intermediate interneurons, M, primary motor neurons.

development in *prdm1a* mutants. This suggests that *prdm1a* and *olig4* are mutual repressors, creating a sharply delineated border by suppressing neighboring cell fate (see model in Fig. 7). This is reminiscent of what is observed in mouse spinal cord development where *Olig3* is required for specification of dorsal class A interneurons (more dorsal dI1–3) and represses the formation of class B interneurons (intermediate dI3–6) (Muller et al., 2005). Class A interneurons are thought to migrate to a more ventral location in the spinal cord, and relay proprioceptive information (Ding et al., 2005; Muller et al., 2005). However, unlike the zebrafish Morpholino phenotype, Olig3$^{-/-}$ mice do not have a defect in FoxD3- and Sox10-expressing neural crest cells, suggesting that Olig3 is required to promote dI1–3 interneuron fate without having an effect on neural crest cell development. RB-like neurons have been characterized morphologically in mammalian embryos but not with molecular markers, thus it is unclear which cells, if any, in the developing mouse nervous system correspond to RB neurons (Humphrey, 1944; Humphrey, 1950). The difference between the function of Olig3/4 in mouse and zebrafish is intriguing and could provide clues to the cell fate relationships between neural crest, RB neurons and interneurons across species. While further studies are necessary, we speculate that RB neurons may have evolved into the more dorsal interneuron that relays proprioceptive information in mammalian embryos.

Notch signaling is an important regulator of cell fate in the nervous system (Artavanis-Tsakonas et al., 1999; Cornell and Eisen, 2005; Lewis, 1998). Its canonical role is in lateral inhibition, where cells within an equivalence domain (cells that are equivalent to each other in developmental potential) signal to each other, and by a stochastic process, one cell becomes a neuron and then inhibits its neighbors from becoming neurons. Notch receptors and delta ligands are expressed at the right time and place to be involved in segregation of cell fate between Rohon–Beard sensory neurons and neural crest cells. In mutations that affect Notch signaling in zebrafish, there is an excess of all primary neurons, including RB sensory neurons, at the expense of trunk neural crest cells (Cornell and Eisen, 2000; Itoh et al., 2003; Jiang et al., 1996). The transcription factors that mediate this process

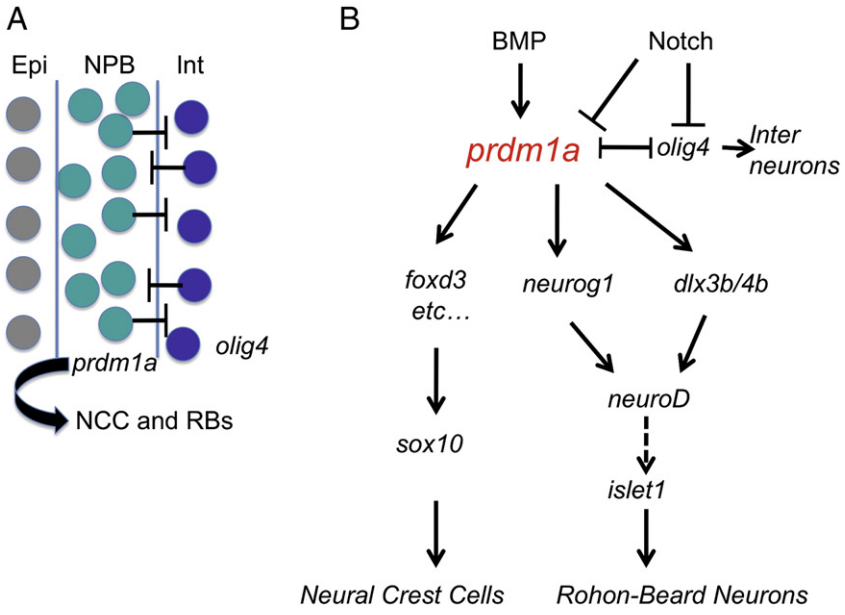

Fig. 7. Model of *prdm1a* function at the neural plate border. (A) Interneurons (Int; blue), Neural plate border (NPB, green) and Epidermis (Epi; grey). Mutual repression between *prdm1a* and *olig4* is required to maintain NCC and RB sensory neuron cell fate. (B) Preliminary gene regulatory network for NCC and RB sensory neuron development.

downstream of Notch include *olig4* and *prdm1a,* which we have shown here to have opposing effects on NPB cell fate specification. While knockdown of *olig4* increases neural crest cells and RB neurons (Filippi et al., 2005), loss of *prdm1a* reduces both cell fates. Because Notch mediates lateral inhibition between these two cell fates, perturbation of Notch signaling results in promotion of neuronal fate at the expense of neural crest fate (Cornell and Eisen, 2005). We then examined what is required for mediating the positive and negative regulation of the Notch signaling pathway on neural crest development. We propose that the mutual repression between *olig4* and *prdm1a* determines the fate of cells of the NPB region. Because *olig4* knockdown can rescue *prdm1a* mutants, *olig4* may be instructive while *prdm1a* is permissive in neural plate border fate. *olig4* knockdown can rescue the neural crest phenotype seen in Notch inhibition, suggesting that *olig4* functions downstream of Notch and normally inhibits neural crest development (Filippi et al., 2005). In the current study, we have shown that Notch can regulate *prdm1a* by normally downregulating its expression. This data is the first to support a role for Notch at two time points in zebrafish neural development: 1) At early stages, during neural induction, to regulate the number of progenitor cells at the neural plate border and, 2) At a later time point in lateral inhibition to promote neuronal fate at the expense of neural crest fate. Interestingly, inhibition of Notch cannot rescue any aspect of the *prdm1a* phenotype, suggesting that *prdm1a* acts downstream of Notch signaling at the neural plate border.

In conclusion, our studies provide further evidence that the transcription factor Prdm1a is a key cell fate regulator at the NPB. In particular, our data indicate that Prdm1a acts by specifically promoting neural crest cell and RB neuron fate, rather than by regulating cell death or proliferation of these cells or their progenitors. We have also shown that *prdm1a* acts downstream of Notch signaling in this cell fate pathway. Finally, *prdm1a* also inhibits *olig4*, thereby repressing the interneuron cell fate within the dorsal spinal cord, and it is the mutually suppressive function of *olig4* and *prdm1a* that regulates neural crest and RB cell fate.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.06.005.

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